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Synthases

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Investigation aimed at understanding the enzymatic mechanism of the two diterpene cyclases, taxadiene and abietadiene synthase, may yield an alternative approach to combinatorial chemistry in the search for new and improved chemotherapeutic and antibiotic drugs. Taxadiene synthase catalyzes the rate limiting step in the conversion of geranylgeranyl diphosphate to taxol. Taxol (Paclitaxel™ Bristol-Myers Squibb) and the related cyclic diterpene, taxotere (Docetaxel™ Rhone-Poulenc) are successfully used as front line chemotherapeutics in the treatment of several types of cancer including breast cancer. Progress was made in establishing assay conditions for the diterpene cyclase and synthesizing novel substrate analogs to be used in investigation of this class of enzymes. The analogs 2,3-dihydro-geranylgeranyl diphosphate and S-geranylgeranyl diphosphorothiolate have been synthesized. Synthesis of verticillene and copalyl diphosphate were not attempted due to potential availability from collaborators. Attempts at synthesizing both substrate analogs 6,7-dihydro-geranylgeranyl diphosphate and geranylgeranyl methylphosphonophosphate were unsuccessful. Several technical hurdles in establishing a reliable enzyme assay were overcome. Transfer of this assay to the rapid quench machine was difficult due to lack of product signal. Lack of signal may be caused by adsorption of the hydrophobic hydrocarbon product to the plastic tubing within the rapid quench machine. This project remains uncompleted but has potential to ultimately yield progress towards its original goals.

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Introduction

Diphosphate bond scission in diterpene cyclase class I enzymes is coupled with generation of a relatively stable allylic carbocation which often leads to formation of a macrocyclic diterpene intermediate. Protonation of a carbon-carbon double bond in class II diterpene cyclases results in formation of a reactive carbocationic species often followed by formation of a bicyclic diterpene intermediate such as copalyl diphosphate. Taxadiene synthase catalyses the rate limiting step in the conversion of geranylgeranyl diphosphate to taxol. Taxadiene is a precursor and branch point to several taxane-based natural products critical for survival of gymnosperm. Taxadiene synthase belongs to the class I diterpene cyclases. An important representative of the class II diterpene cyclases is abietadiene synthase. Abietadiene is the most abundant diterpene in the gymnosperm *Abies grandis* oleoresin. Production of abietadiene is induced during infection by opportunistic predators such as the bark beetle. For many years research in the terpene cyclase field has been focused on understanding these complex cyclase reaction mechanisms which, in the case of abietadiene synthase, comprise up to four different chemical transformations for a single enzyme. The combined approaches of steady state and pre-steady state analyses proposed in this project may ultimately provide novel insights into the mechanisms of these and similar terpene cyclases which to this day have remained elusive using traditional enzymology approaches. In particular, pre-steady state analyses will provide new information regarding key intermediates, rate limiting steps, and active site mechanism which are not detectable through steady state analysis. There is intense investigation of the enzymatic mechanism of many sesquiterpene cyclases whose products include promising antibiotics. In addition, taxol (Paclitaxel[™] Bristol-Myers Squibb) and taxotere (Docetaxel[™] Rhone-Poulenc) are successfully used as chemotherapeutics for several types of cancer including breast cancer. With continued investigation aimed at understanding the complicated mechanisms of these enzymes, an alternative approach to combinatorial chemistry may be realized in the search for new and improved chemotherapeutic and antibiotic drugs. Furthermore, genetic manipulation of the diterpene cyclase mechanism may result in production of novel, complex, and synthetically inaccessible structures with chemotherapeutic or antibiotic potency. The proposed project is thus subdivided into two goals as follows: to define the reaction mechanism of a Class I diterpene cyclase (taxadiene synthase), and to define the reaction mechanism of a Class II diterpene cyclase (abietadiene synthase). Though this project remains unfinished, additional research in this direction may yield the goals originally proposed.

Body

Research accomplishments associated with each task (boxed) outlined in the approved Statement of Work.

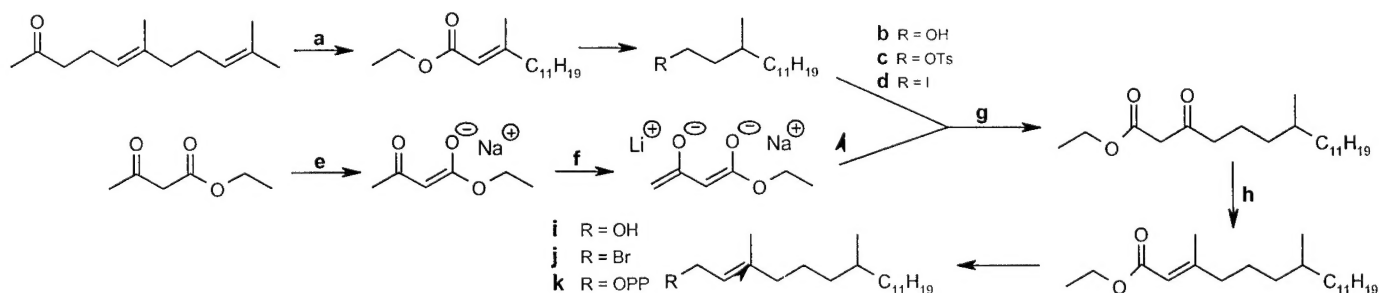
Task 1. Chemical synthesis of enzyme substrates and substrate analogs (months 1-12).

- Synthesis of geranylgeranyl diphosphate and 2,3-dihydro-geranylgeranyl diphosphate (*already complete*)
- Synthesis of 6,7-dihydro-geranylgeranyl diphosphate, geranylgeranyl methylphosphonophosphate, S-geranylgeranyl diphosphorothiolate (months 1-12)

Synthesis of geranylgeranyl diphosphate and 2,3-dihydro-geranylgeranyl diphosphate was completed prior to the submission of this proposal.

Synthesis of 6,7-dihydro-geranylgeranyl diphosphate

The synthesis of 6,7-dihydro-geranylgeranyl diphosphate has so far been unsuccessful. The proposed route of synthesis is illustrated below:



In the following descriptions of reactions, all purified intermediates were structurally confirmed by ¹H NMR. All reactions are performed under nitrogen gas unless described otherwise. In **step a**, geranyl acetone (3 g) was efficiently converted to the corresponding ethyl ester by incubation with equimolar quantities of sodium

hydride (NaH) and triethylphosphonoacetate in benzene overnight at ambient temperature.

Reduction of the ester was performed as illustrated in **step b** by incubation with 4 equivalents of Red-Al and the catalyst copper bromide (CuBr) in 61 ml freshly distilled tetrahydrofuran (THF) under Argon gas. Red-Al was incubated with CuBr for 30 minutes at 0 °C. The reaction was cooled to -78 °C and the ester added (3 g) and the allowed to incubate for another hour at -20 °C. The reaction was quenched with an equal volume of water and ether used for work-up of the reduced ester (2,3-dihydro-farnesoate ethyl ester). Product was resolved from starting material by silica chromatography in 1:32 ethyl acetate:hexanes (yield 80%). This ester was then reduced in one step to the corresponding alcohol by incubation with 2.3 molar equivalents (meq) of lithium aluminum hydride (LiAlH₄, LAH) in 25 ml freshly distilled ether for one hour at 0 °C. The products were resolved on silica gel in 1:32 ethyl acetate:hexanes (yield 76%).

Conversion of the alcohol to the corresponding activated intermediate, 2,3-dihydro-farnesyl tosylate, **step c**, was carried out through incubation with 2.0 meq of recently hexane re-crystallized *p*-toluene sulfonyl chloride (TsCl) and 4(*N,N*-dimethylamino)pyridine in 76.5 ml freshly distilled dichloromethane (CH₂Cl₂) overnight at ambient temperature. Work-up was performed using hexane and 0.1 M sodium hydroxide (NaOH) and 0.1 M hydrogen chloride (HCl). Excess un-reacted TsCl was precipitated by addition of ice-cold hexanes followed by filtration. Products were resolved on silica gel in a solvent system composed of 1:16 ethyl acetate:hexanes (yield 85%, 1.84 grams).

Initially, **step d**, was deferred to determine if the tosylate intermediate would react with the ethyl acetoacetate (EtAcAc) dianion produced in **steps e** and **f**. Formation of the dianion (**steps e** and **f**) was performed using purified EtAcAc. EtAcAc was purified by addition of saturated aqueous sodium bicarbonate (NaHCO₃) and water. The compound was then dried on magnesium sulfate (MgSO₄) and distilled under reduced pressure. NMR analysis was used to confirm the structure and purity of the EtAcAc starting material. Purified EtAcAc (1.65 mmol) was incubated with 1.3 meq NaH in 500 μ l THF at 0 °C for 10 minutes. *n*-Butyl lithium (*n*-BuLi, titrated using diphenyl

acetic acid) was added at 1.1 meq over a period of 5 minutes and allowed to incubate another 10 minutes longer at 0 °C. This solution (300 μ l, 0.33 mmol) was transferred to a second flask containing 2,3-dihydro-farnesyl tosylate (0.33 mmol) in 500 μ l THF and allowed to incubate 30 minutes at ambient temperature after which a second aliquot of 200 μ l dianion was added and the reaction incubated another 30 minutes. Ether and water were added to quench the reaction. Work-up included addition of ether and brine. NMR analysis revealed no well-defined product.

Many attempts were made under various conditions to convert the 2,3-dihydro-farnesyl tosylate to the corresponding iodide (**step d**). A 3 meq excess of sodium iodide (NaI) was added to the dried tosylate compound in acetone and allowed to reflux overnight. Work-up included sodium disulfide (Na₂S₂O₃), water, and brine washes in ether. In pilot studies a discernible iodide spot was observed on silica thin layer chromatography (TLC) in a solvent system of 1:16 ethyl acetate:hexanes using phosphomolybdate to visualize the compounds. However, product yield was very low (yield 20%). Substitution of the ether solvent work-up with CH₂Cl₂ and elimination of a celite filtration step resulted in similar yields. This reaction was attempted at 0 °C and – 20 °C giving similar results and yields. The reaction was repeated in acetone and dimethyl formamide (DMF) solvents which also resulted in similar yields. In another attempt, 5 meq of NaI was added to the tosylate and allowed to incubate at ambient temperature for several hours. Product was recovered; however, the yield was again only 18%. Starting material which did not react with NaI was not able to be recovered.

Some model iodination reactions were performed as a test of the reagents and protocols. Four compounds were individually incubated with 5 meq NaI, including geranyl tosylate, dodecyl tosylate (representing the backbone structure of the parent compound), farnesyl bromide, and farnesyl chloride. Of the four compounds, only the farnesyl chloride and bromide formed the corresponding iodide in good yields. Other model compounds were used to test the effectiveness above the protocol. Using the same protocol as described above, farnesyl bromide, farnesyl chloride, and 1-bromododecane were introduced to separate flasks each containing 4 meq of the EtAcAc dianion (generated as described above) and allowed to incubate at 0 °C for

several hours. Of the three compounds, farnesyl chloride generated the best results in terms of yield of the desired corresponding β -diketone (yield 31%).

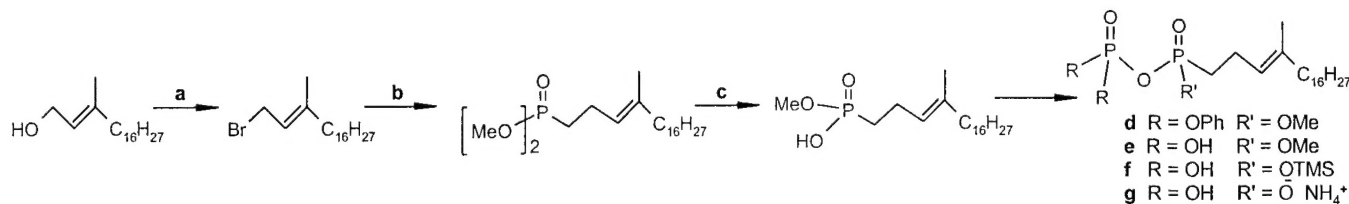
A small amount of 2,3-dihydro-farnesyl iodide was accumulated over several experiments (described above) and used in a pilot study to form the β -diketone ester using the purified EtAcAc dianion as described above (**steps e, f, and g**). NaH (1.2 meq.) was added to 168 μ l freshly distilled THF at -78°C and stirred in the presence of 1.0 meq of EtAcAc for 15 minutes. Then, 1.1 meq of *n*-BuLi were added drop-wise and the reaction allowed to incubate an additional 15 minutes. Farnesyl iodide was then added (1.0 meq) to the flask and the reaction allowed to warm to ambient temperature. On work-up, no product or starting material was detected by NMR or TLC.

Since the model reactions with farnesyl chloride and bromide appeared to fare better, 2,3-dihydro-farnesyl bromide was synthesized using the standard protocols described above. This compound was incubated with roughly 1 meq of the EtAcAc dianion at 0°C for several hours. Several new compounds were generated in the reaction as monitored by silica TLC. On work-up and purification of the various compounds, none proved to be more than degradation products of the starting material.

Similar reactions were attempted in which EtAcAc dianion was substituted for similarly purified methyl acetoacetate dianion in reaction with the 2,3-dihydro-farnesyl bromide. Lithium diisopropyl amide (LDA) was investigated as a substitute for *n*-BuLi. Other variations were examined including order of addition in generation of the dianion (NaH-generated anion added to the LDA, etc) and reduction of reaction temperature to -78°C . None of these yielded detectable amounts of product. Further experiments on larger scale yielded no useful materials with which to proceed with the synthetic route.

Synthesis of Geranylgeranyl Methylphosphonophosphate

Synthesis proceeded as indicated in the proposed synthetic pathway:



Reagents and conditions: (a) Carbon tetrabromide, triphenylphosphine, CH_2Cl_2 ; (b) dimethyl methylphosphonate, THF, *n*-butyllithium, -78°C ; (c) sodium azide, dimethylformamide, 100°C ; (d) diphenyl chlorophosphate, tributylamine, CH_2Cl_2 , rt.; (e) anhydrous tetra-*n*-butylammonium phosphate, pyridine, rt. (f) trimethylsilyl bromide, 2,4,6-collidine, CH_2Cl_2 ; (g) ammonia, methanol.

Carbon tetrabromide (CBr_4 , 1.3 meq) and triphenyl phosphine (P(Ph)_3 , 1.05 meq) were incubated with geranylgeraniol (GGOH) for 3 hours at ambient temperature in freshly distilled CH_2Cl_2 (**step a**). The appearance of product and its structure were confirmed by silica TLC and NMR, respectively. This product, geranylgeranyl bromide was then used directly in **step b** to produce the corresponding methyl ester. In this reaction, 0.21 mmol *n*-BuLi was added to 0.221 mmol dimethyl methylphosphonate (DMMP) at -78°C and allowed to stir for 15 to 20 minutes in 0.5 ml freshly distilled THF. Geranylgeranyl bromide (0.17 mmol) was added to this reaction in 0.66 ml freshly distilled THF slowly, drop-wise and allowed to incubate for 1 hour at -78°C . The reaction was allowed to incubate an additional hour over which time the reaction was warmed to 0°C . The reaction was quenched using 10% HCl after dilution with ether. Work-up included water, saturated NaHCO_3 and brine washes. No product was detected by NMR but the substrate was recoverable. In another attempt at forming the methyl ester, a second flask was used to generate 10 meq of the DMMP anion at -78°C . Aliquots of 1 meq each of the anion were transferred to a second flask containing 1 meq GGBr using a glass syringe cooled with dry ice. Several new substances were detected by silica TLC on addition of 2.5 meq of the anion. The reaction was quenched with 10% acetic acid and the structure of the methyl ester product was confirmed by NMR analysis. The reaction was repeated on a 0.5 gram scale with disappointing yields of 17%. Several additional attempts were made at this reaction with similarly disappointing results.

Synthesis of geranylgeranyl S-thiolodiphosphate (S-geranylgeranyl diphosphorothiolate) was accomplished by graduate student Richard M. Phan while working on a unrelated project funded by an NIH Predoctoral Trainee Grant (GM 08573). Its synthesis is outlined in his recent publication "Synthesis of Isoprenoid S-thiolophosphates as Substrates and Inhibitors for FPPase" (Organic Letters, vol. 2, pp. 2287-2289, 2000).

Task 2. Chemical synthesis of putative intermediates of taxadiene and abietadiene synthase (months 12-18).

- Synthesis of verticillene (months 12-17)
- Synthesis of copalyl diphosphate (month 18)

Neither of these tasks were attempted since it appeared we would be able to obtain at least small quantities of these compounds from collaborators. Instead, the project proceeded to *Task 3*.

Task 3. Kinetic characterization of taxadiene and abietadiene synthase at steady state and development of product assay by HPLC (months 19-27).

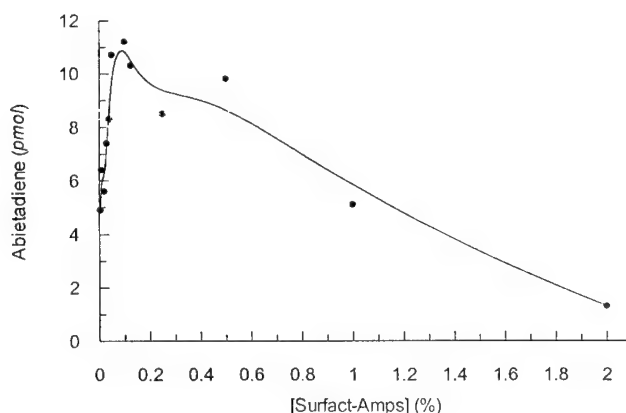
- Testing of various columns and solvent conditions with authentic standards (month 19-20)
- Optimization of steady state enzyme assay (month 20-21)
- Analysis of reaction products using new HPLC assay under various conditions, in the presence of native substrate and substrate analogs to obtain basic kinetic parameters such as K_m , V_{max} , and k_{cat} (month 21-27)

Instead of investigating columns and solvents for product and substrate resolution, a steady state assay was developed to examine the synthases in which the product is extracted from the quench by addition of a layer of hexanes. This quantitative assay will likely work equally well for both synthase enzymes. To date, only *wild type* recombinant abietadiene synthase (rAS) has been studied.

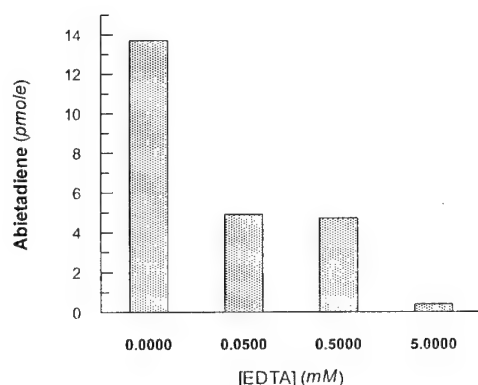
Optimization of the Steady State Abietadiene Synthase Assay

The synthase assay involves incubation of the recombinant enzyme with tritiated geranylgeranyl diphosphate (NENTM Life Science Products, Inc.) at 32 °C. The reaction is quenched using either a KOH:MeOH (1:1) quench with a cold hexanes overlay or just cold hexanes. After vortexing the quenched sample, half of the hexanes overlay is withdrawn using a glass Hamilton syringe and counted in scintillation cocktail. Counts above background represent tritiated abietadiene product.

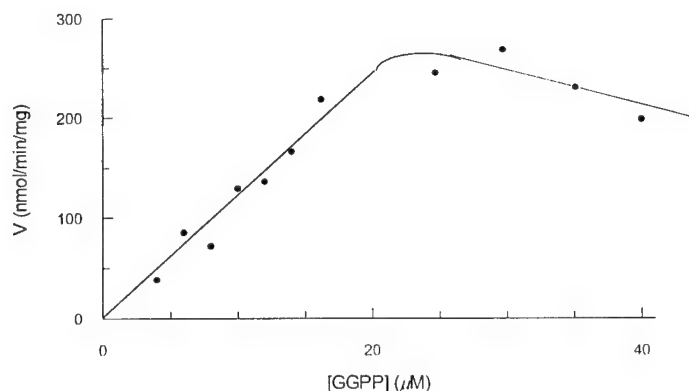
Initial tests showed that the recombinant enzyme yielded 0.2 to 0.6 nmole product per minute in 1 ml assays using a buffer of 0.1 M KCl, 30 mM HEPES, pH 7.24, 7.5 MgCl₂, 20 μ M MnCl₂, 5% glycerol, and 0.1 mg/ml α -casein. Standard assays employed this buffer but at a reduced total volume of 100 μ l or less. During the course of experiments it was discovered that all-glass conditions must be employed due to the ability of the labeled substrate to adhere to all plastics (as much as 40% loss was observed). Glass Hamilton syringes were used to withdraw the hexane layer sample for counting from the quenched reactions. These Hamilton syringes also had to be thoroughly de-contaminated between each experiment since carry-over resulted in significant scattering of data. Siliconized eppendorf tubes were examined as an alternative to the glass vials; however, yields were lower than those recovered using regular plastic eppendorf tubes. Significant substrate inhibition is observed in this enzyme. It was discovered that addition of Surfact-Amps[®] (contains 10% tween-80, Pierce) increased product recovery as shown in the following graph (standard conditions: 3 μ M ³H-GGPP, 100 nM, 2 minutes at 32 °C) (*top of next page*):



Tween was added to a final concentration of 0.1% in all reactions following this experiment. However, addition of isopropanol had no effect on activity up to 5%, beyond 5% isopropanol activity declined sharply. In subsequent experiments it was found that a single extraction of the quenched reaction with an overlay of hexanes was sufficient to quantitatively recover product. Other extraction methods using octanes and pentane gave similar product yields. Addition of EDTA, as predicted, significantly reduced yield of product as shown in the following bar graph (standard conditions, *top of next page*):



The following results are representative of those achieved over many experiments aimed at establishing a reliable steady state abietadiene synthase assay. Once the above mentioned technical factors and problems were discovered and taken into consideration, the following results were obtained (standard conditions) :



There are no data to yet report on the taxadiene synthase enzyme. Studies on taxadiene synthase will commence when the technical problems associated with recovery of product from the rapid quench machine are resolved (*described below*).

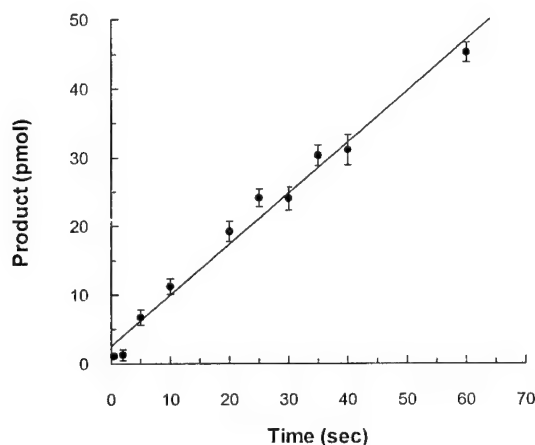
Task 4. Kinetic characterization of taxadiene and abietadiene synthase pre-steady state kinetics (months 28-36).

- Analysis of multiple turnover time courses for both abietadiene and taxadiene synthases under various conditions to determine initial kinetics of reaction (burst phase, rate-limiting steps, etc.) (months 28-30).
- Analysis of single turnover time courses for both abietadiene and taxadiene synthases and attempts to identify trace amounts of putative intermediates using cold trapping methodologies (months 31-36).

A first priority is translation of a working steady state rAS assay to the rapid quench machine; however, the abietadiene synthase assay has not yet been successfully transferred to the rapid quench machine. Assay conditions which appear to work well using the usual glass vials and syringes in a water bath, as described above, yielded no detectable product in the rapid quench machine even when assays were performed with identical conditions (volume, temperature, enzyme concentration, etc) on the same day.

Before use, the rapid quench machine required repairs and usual maintenance including calibration of loop volumes and computer parameters used in calculating ejection volumes (the computer controls the step-motor which drives the solutions through the rapid quench tubing and out the exit loop). After these preparative steps were accomplished, a known reaction was used to confirm the rapid quench is operating within standard acceptable parameters. The test enzyme used was farnesyl diphosphate synthase (a sesquiterpene synthase) which catalyzes the transformation of geranyl diphosphate and isoprene diphosphate to farnesyl diphosphate. This enzyme is well characterized and standard parameters for its investigation by rapid quench have been previously reported. Using [FPP] of 0.5 μ M, [GPP] of 50 μ M, and [IPP] of 12.5

μM , time points of zero through 60 seconds were assayed in triplicate at 4 $^{\circ}\text{C}$. Assay buffer and quench have been described elsewhere. The data were consistent with that reported and observed previously using this instrument under these conditions:



These data indicate that the rapid quench is functioning normally. The following experiments were performed on the rapid quench machine with rAS. Standard assays included 0.3 μM rAS and 3 μM ^3H -GGPP (740 dpm/pmol, or 4440 dpm per turnover). Many of the first experiments generated scattered data that was inconclusive because some of the details discovered (above) about the assay were not yet known. After several unsuccessful attempts to detect product at 3 and 6 μM ^3H -GGPP, several issues were investigated:

- 1) the scintillation counter was tested for accuracy and calibrated
- 2) large scintillation and small scintillation vials functioned identically in the counter
- 3) acid hydrolysis examination of the radiolabeled substrate revealed predicted activity
- 4) several negative control experiments were assayed (no enzyme present in the reactions) which revealed that these controls were reproducible

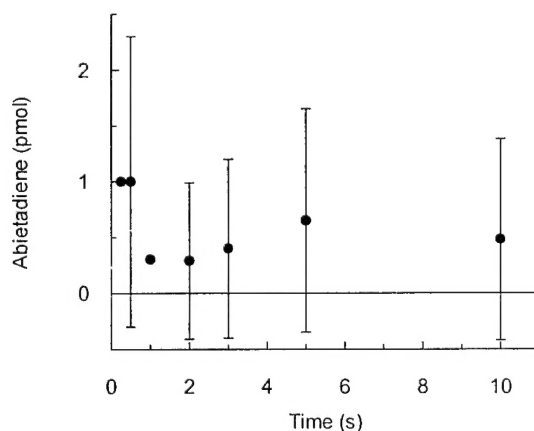
Injection of a known quantity of label into the rapid quench and quantitating the recoverable activity suggested that as much as 30% of the substrate label was being lost. After this discovery, several other possibilities were examined:

- 1) An aliquot of water from the water bath which circulates through a plexiglass box enclosing the rapid quench was assayed for contamination of radioactivity. None was found indicating there are no detectable internal leaks.
- 2) Various solvent washes were examined for ability to recover the label including dimethyl formamide, hexanes, acetone, and methanol. None were able to fully recover 100% of a standard radiolabel injected into the rapid quench.
- 3) Injection of a standard known amount of the radiolabel into a small section of the tubing used within the rapid quench adsorbed as much as 30% of the radiolabel.
- 4) 100% of a known standard of ^3H -FPP standard was recovered from the same tubing.
- 5) Enzyme exposure to and recovery from of the rapid quench did not reduce the enzyme activity by more than 10% when compared with controls which were not exposed to the rapid quench.

After consideration of the results of these controls it was concluded that a loss of 30% was tolerable and data would have to be adjusted to reflect this loss in the final analysis. Many other tests were performed on the rapid quench machine in which glass tube and rapid quench assays were performed in parallel at the same time and under identical reaction conditions; however, the results from the rapid quench machine were too scattered to be interpreted. Where in the glass vial experiments a typical rate of product formation was observed, no product was observed exiting from the rapid quench machine. The following tests were performed:

- 1) The elution buffer was changed to one containing 1% tween and 10% isopropanol or 50% methanol. No counts above background were detected.
- 2) The ^3H -GGPP label from NEN was examined by thin layer chromatography, HPLC, and acid lability tests. Results of these tests were consistent with the known properties of geranylgeranyl diphosphate.

One last experiment was performed in which the maximum amount of enzyme available and radiolabel specific activity were used in order to boost signal. In this last experiment, the specific activity was increased to 17,930 dpm/pmol GGPP and 1.5 μ M rAS enzyme. The following data were obtained:



It is evident from these data that no meaningful conclusions can be drawn from the experiments until the technical difficulties surrounding recovery of the 3 H-abietadiene product can be resolved. This compound is highly hydrophobic and may be adhering to the tubing within the rapid quench. The following potential solutions to this technical problem may be examined in the future:

- 1) It is possible that different buffer additives, either in the reaction or in the washes, can be used to recover enough signal from the experiment.
- 2) It may be possible to quantitate the disappearance of substrate rather than appearance of product.
- 3) Use of much higher concentrations of enzyme (higher than 1.5 μ M) and larger quantity of radiolabel per turnover may enable detection of product.

Furthermore, the Croteau group, the collaborator which has been supplying recombinant enzyme for these studies, have reported the genetic engineering of abietadiene synthase. Several mutants have been recently reported which may be beneficial to this project and may pose a solution to some of the technical problems. One aspect of rAS which limits our ability to study the enzyme is its reported substrate inhibition. One of the single-site mutants of rAS eliminates the second cyclization

reaction from the mechanism (e.g. conversion of copalyl diphosphate to abietadiene). This mutant displays none of the substrate inhibition properties observed in the *wild type* enzyme. A second single-site mutant is able to only catalyze the second half of the cyclization process (e.g. conversion of geranylgeranyl diphosphate to copalyl diphosphate). These two mutants will allow isolated mechanistic investigation of both half reactions and may overcome the inability to detect product by allowing use of much higher concentrations of substrate. These results have been reported in the following paper: "Bifunctional Abietadiene Synthase: Free Diffusive Transfer of the (+)-Copalyl Diphosphate Intermediate Between Two Distinct Active Sites" (*submitted to J. of the American Chemical Society, 2001*).

Key Research Accomplishments

- Synthesis of the analog 2,3-dihydro-geranylgeranyl diphosphate was completed
- Synthesis of the analog S-geranylgeranyl diphosphorothiolate was completed
- Abietadiene synthase steady state assay was optimized
- Initial hurdles in analysis of diterpene hydrocarbons using a rapid quench technique have been investigated

Reportable Outcomes

There are no clearly reportable outcomes. No manuscripts, abstracts, presentations, patents, or licenses applied for and/or issued resulted from this work. No degrees were obtained through the support of this award. No cell lines were developed. No tissue or serum repositories were created. No informatics such as databases and animal models, etc. resulted from this work. No additional funding was applied for based on work supported by this award. No employment or research opportunities were applied for or received based on the experience and training supported by this award.

Personnel

Thomas J. Siepmann, Ph.D. was the sole recipient of funding from this award with the University of Utah being the secondary recipient of supporting funds in the form of indirect overhead costs. Thomas J. Siepmann, Ph.D. is now a full time student at the University of Dayton School of Law in Dayton, Ohio pursuing a career in intellectual property and patent law.

Conclusions

Though initial attempts at synthesis of several of the substrate analogs was fruitless, several alternate synthetic routes remain to be investigated. A consistent steady state assay for abietadiene synthase took several months to establish; however, this step was necessary for the study of both synthases at steady state and pre-steady state. The groundwork was which will allow rapid analysis of these enzymes in future studies was established. Technical difficulties in working with the rapid quench appear at least temporarily insurmountable. The recent generation and study of single-site mutants of abietadiene synthase may prove extremely useful in future mechanistic studies of this enzyme and may be necessary in resolving the technical problems associated with the rapid quench. Rather than adhere strictly to the timelines proposed in the Statement of Work, it was decided to focus first on a single enzyme to establish the necessary assays. Although this project only lasted one year, out of a proposed three years of study, many of the necessary preliminary studies were completed which will allow further progress in the study of both enzymes should someone continue the project in the future. Future work, if the project is continued, should include initiation of taxadiene synthase assays, further examination of alternate synthetic routes for synthesis of 6,7-dihydro-geranylgeranyl diphosphate, and investigation of the performance of single-site mutants of rAS in pre-steady state assays.